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Effect of *Nannochloropsis salina* on the survival and growth of phyllosoma of the tropical spiny lobster, *Panulirus homarus* L. under laboratory conditions

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Abstract

The first successful advanced (stage VIII) larval development of the tropical spiny lobster, *Panulirus homarus* L. under laboratory culture has been achieved in a short span of 42 days. The culture treatments were maintained in two different systems, one with microalgae, *Nannochloropsis salina* (30-40 million cells/mL) and the other without microalgae. Phyllosoma attained stage VIII (5.25 mm) and stage V (3.36 mm) with an intermoult period of 4-6 and 5-9 days in the two systems respectively. The larvae moulted nine and six times in the microalgal and non-algal systems, respectively. Phyllosoma were fed with freshly hatched live *Artemia salina* nauplii (0.32 mm) for an initial period of 10 days, followed by juvenile *Artemia* (1.45 –1.54 mm) enriched with microalgae for the subsequent 15 days. *Artemia* enriched with commercial polyunsaturated fatty acid concentrate (DHA-Selco) was fed to the larvae for the rest of the culture period. Salinity and temperature were maintained at 33 ± 2 ppt and 28 ± 2 ^{0}C, respectively, while the pH recorded an optimum of 7.9 ± 0.5. The microalgal larval rearing system had lower bacterial load (total heterotrophic marine count, 1.0 X 10^3 CFU m L^{-1} and total *Vibrio* sp. count, 20 CFU/mL) compared to the non-microalgal system.

Keywords: Spiny lobster, *Panulirus homarus,* phyllosoma, *Artemia, Nannochloropsis salina*

Introduction

Spiny lobsters (Decapoda, Palinuridae) are one of the world's most economically valuable seafoods and form the basis of important fisheries worldwide (Jeffs and Hooker, 2000). Increasing global demand, high product value and recent concern over the sustainability of wild stocks have prompted research into commercial hatchery production of larvae for both replenishment of wild stocks and commercial ongrowing. To ensure future expansion and sustainability of spiny lobster aquaculture, hatchery technology for seed production needs to be developed and for which the phyllosoma larvae need to be cultured through their entire larval phase to the puerulus stage (Crear *et al*., 1998). Among the twelve species of lobsters distributed along the Indian coast, only four species of spiny lobsters (three littoral and one deep-sea) and one species of slipper lobster are commercially important (Radhakrishnan *et al*., 2005). The spiny lobster *Panulirus homarus,* the major species distributed along the southern part of the Indian coast is a potential species for aquaculture (Radhakrishnan and Vijayakumaran, 2004).

Although part of the market demand may be met by grow-out puerulii collected from the wild, the primary goal of the industry is development of closed-life system. Commercial farming of lobsters is still not feasible because of difficulties in rearing the delicate phyllosoma through their prolonged

larval phases. Depending on the species, the larval phase may range from 4 to 12 months and takes place in oligotrophic water environment with the phyllosoma larvae having between 6 to 15 morphological stages and a final planktonic larval puerulus stage (Kittaka and Abrunhosa, 1997).

Artemia nauplii are reported to be the most suitable feed for early stage phyllosoma (Carlberg and Van Olst, 1976; Vijayakumaran and Radhakrishnan, 1986; Kanazawa, 1994) and have been used effectively as the major or sole component for culture of phyllosoma larvae (Kittaka, 1994 a; Tong *et al*., 1997; Moss *et al*., 2000; Ritar *et al*., 2002, 2003). Phyllosoma of *J. verreauxi* and *J. edwardsii* were found to prefer nauplii in the initial two stages and *Artemia* juveniles in later stages (Kittaka, 1994 b; Ritar *et al*., 2003). Nutritional enrichment of *Artemia* has been tried to improve larval survival rates (Nishimura, 1983). The biochemical composition of *Artemia* is regarded as important in optimizing larval nutrition for survival and growth of aquaculture species (McEvoy and Sargent, 1998; Narciso *et al*., 1999). Enrichment procedures and products have been developed to improve the nutritional quality of *Artemia* nauplii and metanauplii (Rees *et al*., 1994; McEvoy and Sargent, 1998; Sorgeloos *et al*., 2003). In New Zealand, stage I to stage VIII phyllosoma larvae of *J. edwardsii* were fed with 2–3 mm long *Artemia* enriched with microalgae (Booth, 1996). Few temperate species of spiny lobster larvae have been successfully cultured in a microalgal system (Kittaka *et al.,* 2005). This idea of larval feeding method originated from the 'ecosystem' culture method (Hudinaga and Kittaka, 1967), which was developed earlier for penaeid shrimp. Major problems in successful culture of spiny lobster phyllosoma are the lack of information on larval diet, feeding habits (Mikami *et al*., 1994), and difficulty in maintaining optimal water quality throughout the culture period (Kittaka, 1997 a). In an earlier investigation, *P. homarus* phyllosoma larvae were reared to stage VI in 60 days on an exclusive diet of freshly hatched *Artemia* nauplii (Radhakrishnan and Vijayakumaran, 1995).

In the present study, an attempt was made to investigate the effects of the microalga, *Nannochloropsis salina* on improved survival and growth of phyllosoma larvae under laboratory culture conditions. The larvae were maintained in two different culture systems one with the microalga *N. salina* and the other without microalga. Various environmental and microbiological parameters were compared in these two different systems and they were correlated with the growth and development of the phyllosoma of *P. homarus.*

Material and methods

***Broodstock capture and larval handling*:** The adult *P. homarus* broodstock was collected from coastal waters of west coast of India (08.09^0 N lat. 77.35^0 E long.) at a depth of 10-20 m. The broodstock was maintained in outdoor circular holding tanks (10,000 L, 4 m diameter) containing UV-filtered (1 µm) seawater (FS) maintained at temperature varying between 26 and 30^{o}C, salinity from 33 to 35 ppt and pH 7.5-8.5. During breeding season (December to March), the females were examined for the presence of eggs, and in those carrying eggs, the estimated date of hatching was determined by inspecting the development of embryo. Berried female with ready-to-hatch (dark brown coloured eggs) was removed from the holding tank and transferred to hatching tank of capacity 5000 L. Soon after hatching, the female was removed and newly hatched phyllosoma larvae were transferred to a larval-rearing tank containing 200 L of seawater. The larvae obtained from a single female (carapace length 100 mm; total length 315 mm) were used in this experiment. The larval-rearing tank was previously chlorinated, sundried and then filled with fresh microfiltered and chlorinated seawater. The seawater used for larval rearing was regularly treated with chlorine (990 mL, 100 g L^{-1} stock) for 24 h followed by neutralization with sodium thiosulphate (1M, 300 mL) and was aerated vigorously. The larval-rearing tank received 30% water exchange for every 24 h with fresh seawater.

***Larval rearing*:** Healthy larvae stocked in cylindro-conical, non-transparent FRP tanks with white inner surface were kept under subdued sunlight. The stocking density was 10 larvae/L. In one set of tanks, the culture of *N. salina* (30-40 million cells mL^{-1}) was added 4 days before the

addition of phyllosoma while the other was kept as control without adding microalgae (hereafter termed as algal and non-algal systems). Algal system was regularly replenished with *N. salina* to maintain the cell concentration. Environmental parameters (temperature, salinity, and pH) were recorded daily. Water quality parameters *viz*., dissolved oxygen (DO), ammonia (NH_3) and chemical oxygen demand (COD) were analyzed once in a week. Seawater used for phyllosoma culture was filtered through a 1µ cartridge filter and irradiated with UV light. Bacteriological analysis was carried out as per the standard procedure (USFDA, 2001). Total heterotrophic marine counts and total *Vibrio* sp. counts were obtained by using Zobells' marine agar and TCBS agar, respectively. Partial exchange (~30%) of water was carried out daily and the phyllosoma larvae were maintained in the same tank throughout the culture system.

Diets for rearing phyllosoma

Production of Nannochloropsis salina: *N. salina* stock was cultured in Conway's medium (Walne, 1979; Gopinathan, 2000). The inoculated tubes and flasks were kept under light intensity of 3000 lux (LT Lutron, LX, 101 Luxmeter) at a temperature of 27-30°C. The growth of microalgae was observed daily and the cell concentration (30-40 million cells mL^{-1}) was monitored every alternate day using haemocytometer. Mass culture of microalgae was carried out in a circular FRP tank under the light intensity (6000 lux) during peak hours of sunlight, and provided with vigorous aeration. Nutrient mixture (urea 4 g; groundnut oil cake 4 g; super phosphate 10 g) was added to seawater (1 ton). The pH, dissolved oxygen (DO), temperature and salinity were maintained between 7.5-8.3, 4.1-5.5 mg/L, 25-34°C and 28-33 ‰, respectively in the tanks.

Artemia production and enrichment: Encapsulated *Artemia* cysts (Brine shrimp eggs, pro 80™, USA) (0.5- 1.0 gm) were hydrated and disinfected in 0.4% aqueous sodium hypochlorite (w/v) solution for 2 h. After disinfection, the cysts were washed extensively on 120 µm screen with fresh seawater until traces of chlorine were removed. After dechlorination, the cysts were hatched in fresh sterilized seawater at 27-29^{0}C and 28-32 ppt salinity under gentle aeration. Freshly hatched nauplii were harvested after 18 h, separated from hatching debris and unhatched cysts, and once again thoroughly rinsed with seawater on 120 µm screen. The washed nauplii (0.2-0.3 mm) were then resuspended in seawater and fed to phyllosoma at a feeding density of one nauplii/ml for the initial 10 days. Juvenile *Artemia* (1.4 –1.54 mm, 5 days old) fed on *N. salina* were given as feed for the next 15 days and sub-adult *Artemia* maintained on *N. salina* and further enriched with polyunsaturated fatty acid (PUFA, DHA-Selco for 2 h) were fed for the following 15 days of culture period. Prior to use, *Artemia* were disinfected in a formalin bath (100 mg/L for 10 min), and rinsed on a 250-µm screen with fresh seawater. *Artemia* were replaced daily because of the decline in their nutritive value. This was achieved by replacing the fine screen with coarse screen to allow passive flushing of all *Artemia* in 1–2 h from the vessels without causing damage to the larvae. Dead larvae, exuviae, excess food and debris were removed daily by siphoning to maintain good water quality.

***Morphological measurements and survival of phyllosoma larvae*:** Total body length (from tip of the cephalothorax to the posterior point of the abdomen) was measured in 10 randomly chosen newly hatched Stage I phyllosoma. The data were collected at every moult for both the tanks. Measurements (n=10) were obtained using a binocular microscope fitted with micrometer at 40X (Model No. SZ –PT, Olympus, Japan). The survival of phyllosoma as a percentage of the entire population was measured in microalgal and non-algal systems. Since it was difficult to estimate the larval survival with low densities of unevenly mixed larvae, the same was calculated based on counts of dead animals siphoned daily from the bottom of each larval-rearing tank.

***Bacteriological quality*:** Sample of the culture water was diluted serially in sterile seawater and each diluted sample was spread in duplicate on the petriplates (90 mm dia) preset with ZoBell's 2216 agar (Difco Laboratories, Detroit, USA) and TCBS (thiosulfate citrate bile sucrose) media. ZoBell's marine agar was used to isolate aerobic heterotrophic

marine bacteria while TCBS was used as a selective media for *Vibrio* sp. (Bolinches *et al*., 1988). The inoculated plates were incubated at 37°C and total counts of cultured bacteria were recorded based on the recognizable colony characters (Igarashi *et al*., 1990; Igarashi *et al*., 1991) after 24-36 h as colony-forming units (CFU mL^{-1}). Total counts were recorded from plates ranging 30 to 300 colonies. No further colonies were visible with extended period of incubation for 72 h.

Results

***Morphological changes and per cent survival of phyllosoma larvae*:** Phyllosoma larvae were found to be actively swimming towards light (positively phototactic). The larvae moulted once at stage I through stage VI, but at stage VII and VIII one additional sub-stage was recorded in the microalgal system. Larvae moulted to Stage II in 5 to 6 days after hatching. The intermoult interval of phyllosoma larvae was found to increase in both the tanks as the morphological stages progressed from stage II onwards. In the non-algal system, the larvae moulted once at stages I and II, with one sub-stage at stage III; and two sub-stages at stages IV and V. The intermoult interval of phyllosoma larvae reared in the non-algal system was found to be longer than in the microalgal system. Phyllosoma larvae moulted 9 and 6 times, each with an intermoult period of 4-6 and 5-9 days attaining stage VIII and stage V respectively in microalgal and non-algal tanks.

The mean size of the post-hatched phyllosoma was 1.33 ± 0.01 mm (n=10). The phyllosoma larvae in the algal system reached the stage V after 22 days with a mean size of 4.40 ± 0.05 mm, whereas in the non-algal system the same stage was attained after 42 days, having a size of 3.36 ± 0.03 mm. Phyllosoma survived upto stage V in the non-algal system and upto stage VIII (5.25 ± 0.04 mm) in the algal system after 42 days with 33% survival (Table 1).

The differentiation of various stages (stage I to stage VIII) was carried out on the basis of morphology *viz*., developments in pereiopods, segmentation of eyestalk and antennae. In stage I larvae, the eyestalk was unsegmented, whereas in stage II it became segmented. The exopod of third pereiopod became setose in stage III. The fourth pereiopod, which appears in stage III, becomes

Table 1. Comparison of different dimensions (μm) of *P. homarus*, phyllosoma in algal and non-algal systems

Stages	[a]TL (mm)	[b]CL (mm)	[c]THL (mm)	[d]CW (mm)	[e]ABD (mm)
Algal system					
I	1.33 ± 0.01	0.73 ± 0.02	0.50 ± 0.02	0.73 ±0.01	0.25 ± 0.02
II	1.43 ± 1.38	0.83 ± 0.03	0.38 ± 0.01	0.75 ± 0.03	0.25 ± 0.01
III	2.62 ± 0.06	1.48 ± 0.01	0.80 ± 0.01	1.23 ± 0.01	0.29 ± 0.01
IV	2.68 ± 0.10	1.28 ± 0.03	0.74 ± 0.03	1.28 ± 0.05	0.34 ± 0.01
V	4.40 ± 0.05	2.80 ± 0.08	1.15 ± 0.33	1.75 ± 0.02	0.60 ± 0.03
VI	4.60 ± 0.06	2.96 ± 0.04	1.18 ± 0.01	2.16 ± 0.04	0.64 ± 0.02
VII a	4.80±0.01	3.12±0.05	1.21±0.30	2.22±0.02	0.69±0.04
VII	5.10±0.02	3.41±0.03	1.25±0.01	2.31±0.03	0.74±0.01
VIII a	5.25 ± 0.02	3.75 ± 0.02	1.29 ± 0.02	2.35 ± 0.06	0.55 ± 0.01
VIII	5.25 ± 0.04	3.75 ± 0.04	1.33 ± 0.02	2.35 ± 0.06	0.55 ± 0.03
Non-algal system					
I	1.33 ± 0.01	0.73 ± 0.03	0.50 ± 0.01	0.73 ± 0.02	0.25 ± 0.01
II	1.36 ± 0.02	0.83 ± 0.02	0.38 ± 0.04	0.75 ± 0.01	0.25 ± 0.02
III	2.13 ± 0.01	1.20 ± 0.04	0.68 ± 0.02	1.23 ± 0.03	0.27 ± 0.01
IV a	2.23±0.03	1.24±0.01	0.70 ± 0.02	1.28 ± 0.04	0.28 ± 0.01
IV	2.43±0.02	1.31±0.02	0.76±0.03	1.32±0.02	0.31±0.03
V a	2.72 ± 0.03	1.46 ± 0.01	0.82 ± 0.02	1.48 ± 0.04	0.34 ± 0.01
V	3.36 ± 0.03	1.87 ± 0.06	0.95 ± 0.01	1.75 ± 0.02	0.36 ± 0.03

[a] TL = total length; [b] CL = cephalic shield length; [c] THL = thoracic length; [d] CW = carapace width; [e] ABD = abdomen length; dimensions were measured in duplicate and standard deviation was recorded (n = 10)

biramous in stage IV and in stage V, the exopod of fourth pereiopod appeared to become setose (Fig. 1A). In stage VI, the antennule becomes four-segmented, in stage VII 5th periopod appeared as small bud, in stage VIII a, 5th pereiopod budding becomes elongated and in stage VIII, 5th pereiopod becomes biramous (Fig.1B) and Fig. 1C shows the oval cephalothorax of phyllosoma of the same stage.

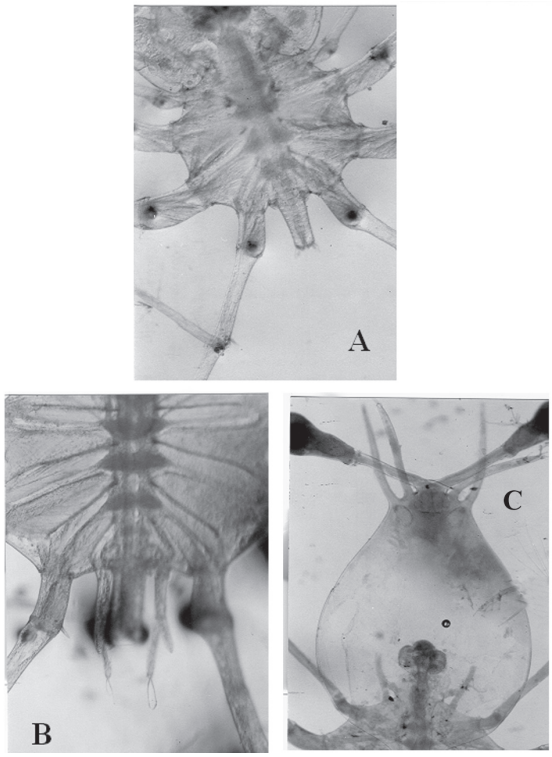

Fig. 1. Light microscopy picture of *P. homarus* phyllosoma, showing external morphology: A) V stage phyllosoma showing 4th biramous pereiopod (non-algal system), B) Abdominal portion of VIII stage phyllosoma (microalgal system), C) VIII stage phyllosoma with oval cephalothorax (microalgal system)

***Water quality*:** In about two weeks, the density of *N. salina*, increased 5 to 10 times in the culture system from 1-2 million cells/mL to 5-10 million cells/mL. During this time, early and mid-stage phyllosoma fed actively on *Artemia* nauplii and survival was found to be as high as 85% in the algal and 60% in non-algal systems. Environmental parameters were found to be optimum in both the tanks. The Chemical Oxygen Demand (COD), ammonia-N, and dissolved oxygen (DO) were used as indicators of water quality. The maximum COD and ammonia-N were 0.76 mg/L and 0.35 mg/L respectively in the microalgal tanks, while in the non-algal tanks, the corresponding values were found to be as high as 1.93 mg/L and 0.68 mg/L. DO ranged from 4.5-6.5 and 4.1-5.3 mg/L, and ammonia-N was found to be 0.12-0.35 and 0.14-0.68 mg/L in algal and non-algal tanks, respectively.

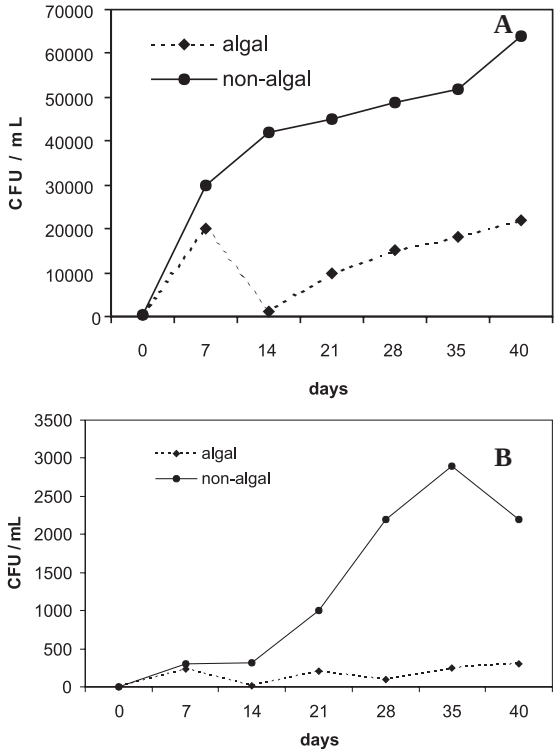

Fig. 2. A comparison of total heterotrophic marine bacterial count (A) and *Vibrio* counts (B)

***Microflora*:** The initial count of marine bacteria, and *Vibrio* sp. were found to be 2 * 100 CFU mL^{-1} and 2 CFU mL^{-1} respectively, in both the tanks. Total heterotrophic marine bacterial (HMB) and *Vibrio* counts (VC) were found to be higher ($2.0*10^4$ CFU mL^{-1} and $2.3*10^2$ CFU mL^{-1}) in the initial period of 5 days and then exhibited a declining phase ($1.0*10^3$ CFU/mL - 20 CFU. mL^{-1}) during 14 days of culture period in the algal sytem. At the later phase of culture, the HMB and VC counts

increased to $2.0*10^4$ CFU mL^{-1} and $3.0*10^2$ CFU mL^{-1}, respectively in the algal tanks. The HMB and VC exhibited an incremental trend throughout the culture period in the non-algal system (Fig. 2). During the last phase of culture period, the tanks were found to be infested with *Lyngbya* sp., a bluegreen alga. The long delicate pereiopods of phyllosoma got entangled with these filamentous algae resulting in larval mortality and collapse of the culture systems (Fig. 3).

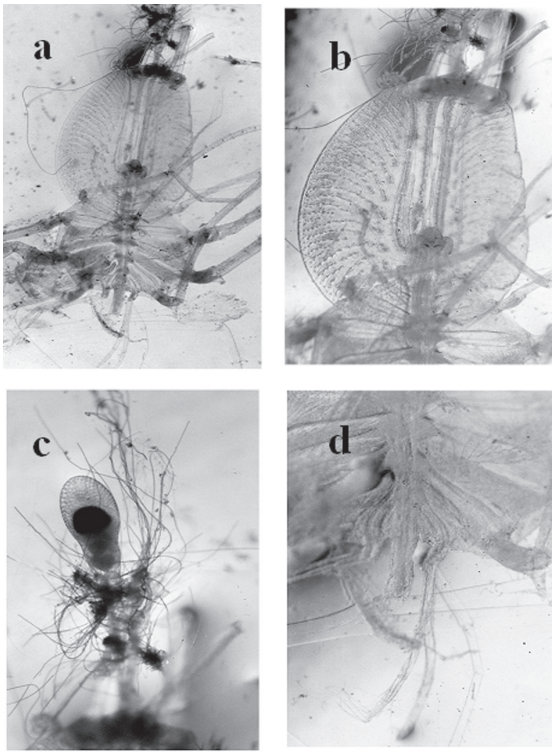

Fig. 3 Light microscopy picture of *P. homarus* phyllosoma, showing external morphology: a) full view stage VIII phyllosoma entangled with bluegreen algae (*Lyngbya* sp.), b) stage VIII phyllosoma cephalic region entangled with bluegreen algae, c) eye dislodged from the phyllosoma, d) pereiopods dislodged from phyllosoma due to algal attack

Discussion

In earlier studies (Radhakrishnan and Vijayakumaran, 1995) low survival of *Panulirus homarus* phyllosoma in laboratory culture condition was attributed to poor water quality and nutrient deficiency. In this study, inclusion of the microalga *N. salina* in the culture tank of phyllosoma has apparently resulted in increased larval growth, higher survival and reduced intermoult period. Further it has contributed to the increased nutritional value of the *Artemia* consumed by the phyllosoma as the algae contained a range of nutrients such as PUFA, vitamins and growth factors (Hirayama *et al*., 1995; Lubzens *et al*., 1995). The microalga appears to provide the phyllosoma with essential micronutrients, and exogenous enzymes facilitating digestion during early larval stages (Dhert *et al*., 1993). Proximate composition analyses of *N. salina* revealed high ash content (32.5% on wet weight basis) which may have possibly contributed to the mineral supplementation of the young larvae. Feeding *Artemia* at an optimum density is important in managing large-scale phyllosoma culture systems not only to maximize survival and growth, but also to minimize costs (Vijayakumaran and Radhakrishnan, 1986; Tong *et al*., 1997; Moss *et al*., 1999).

In the microalgal system there were no intermediate stages discernable from stage I through stage VI, but a single sub-stage was present at stage VII and VIII. In the non-algal system, one sub-stage was noticed at stage III and 2 at stages IV and V. In this study phyllosoma of *P. homarus* attained stage VIII in 42 days in contrast to stage VI in 60 days as reported earlier (Radhakrishnan and Vijayakumaran, 1995). The phyllosoma larvae of the spiny lobster, *Panulirus japonicus* were reared to stage VIII in a longer stretch of 200 days by Kittaka and Kimura (1989). The mid-phase of culture period exhibited high bacterial load in non-algal system (total heterotrophic marine bacterial count, $6.4*10^4$ CFU mL^{-1} and *Vibrio* count, $2.2*10^3$ CFU mL^{-1}) while in algal tanks there was a sharp decrease in total heterotrophic bacteria count to 1000 CFU mL^{-1} and total *Vibrio* count to 20 CFU mL^{-1} after stage IV (14 days). The decrease in the bacterial load in the microalgal system suggests that bacteria might have been controlled by the propagation of *N. salina*. This result supports the report of Austin *et al*. (1992) that microalgae have unique antibacterial and/or immunostimulatory properties. Regular supplementation of microalgae

prevented the outbreak of pathogenic bacterial infections. Low bacterial count in microalgal system was attributed to the antibacterial polysaccharides present in the cell wall of the algae which stimulate the non-specific immune system in these early larval stages and also appear to affect the feeding activity of the phyllosoma larvae (Igarashi *et al*., 1990). Improved survival of early stage larvae of *Panulirus japonicus* was achieved on culturing of phyllosoma larvae with *Nannochloropsis* sp. (Kittaka, 1991). Kittaka (1994b) stated that presence of microalgae in rearing tanks not only inhibited proliferation of ciliate contamination, but also served as a natural water purifier, enhancing survival rate, moulting frequency and growth of the larvae. In the present study the bacterial load was found to be significantly reduced in the microalgal system, possibly due to the buffering effect of *N. salina*.

Use of live feed (*Artemia*, 1-2 mm) in rearing systems was not only found to be more suitable for feeding the larvae, but also helps in keeping up the water quality as compared to the use of chopped mussel meat. In the present study use of *Artemia* (5 days old) enriched with DHA-Selco for 2 h was found to be efficient for rearing phyllosoma grown in microalgal water, contributing to their faster development as compared to that in non-algal system. Moreover larger phyllosoma prefer to catch bigger size *Artemia* with their well-developed pereiopods (Illingworth *et al*., 1997).

The Chemical Oxygen Demand (COD) and ammonia-N, which are the indicators of water quality, were in check in the microalgal sytem. *N. salina* appeared to act as water conditioner possibly by stripping off nitrogenous substances, or by changing the properties of the incoming light (Kittaka *et al*., 2005). Though UV treated seawater was used for carrying out all the operations in the hatchery, probability still remains for the contamination of seawater from air-borne spores of alga. The two sets of tanks were found to be infested with toxic bluegreen alga, *Lyngbya* sp. The alga gradually proliferated as a dense mat on the sides and bottom of the tanks as the tanks were kept undisturbed. Mortality of stage VIII phyllosoma in the algal and stage V phyllosoma larvae in the non-algal tanks appeared to be due to the combining effect of larval entanglement among the algal strands and depletion of water quality due to the decomposition of *Lyngbya*. However, the results are significant because of faster growth and shorter intermoult duration of the larvae which are presumably due to nutritional enrichment of food and improved water quality of the microalgal system. The major factor of larval mortality might have occurred due to the injury to long pereiopods by entangling with the slimy filamentous *Lyngbya* sp. The lost pereiopods regenerated at the following moult, but the regenerated larvae were fragile, and often lost the pereiopods repeatedly, leading to decreased feeding and finally to mortality.

Culture of decapod larvae along with microalgae has been reported to be effective in controlling water quality (Hudinaga and Kittaka, 1967; Kittaka, 1997). The fast growth and high survival of early stage phyllosoma larvae of *P. homarus* in culture system circulated with *N. salina* was due to i) maintenance of improved water quality, ii) continuous enrichment of *Artemia* by feeding on *N. salina* in the microalgal system and iii) avoidance of frequent larval transfer to new tanks. However, from the present experiment, it is clear that culture of phyllosoma larvae for the entire larval phase may not be feasible in the same system as the epiphytic growth is likely to interfere in maintaining a healthy environment which is the prerequisite for larval culture of lobsters.

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